

**IDENTIFICATION OF A NOVEL PHYTOESTROGEN:
TRIGONELLINE**

A Senior Scholars Thesis

by

KATARINA MARIE YACKLEY

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2008

Major: Nutritional Sciences

**IDENTIFICATION OF A NOVEL PHYTOESTROGEN:
TRIGONELLINE**

A Senior Scholars Thesis

by

KATARINA MARIE YACKLEY

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

Approved by:

Research Advisor:
Associate Dean for Undergraduate Research:

Clinton Allred
Robert C. Webb

April 2008

Major: Nutritional Sciences

ABSTRACT

Identification of a Novel Phytoestrogen: Trigonelline (April 2008)

Katarina Marie Yackley
Department of Nutrition and Food Science
Texas A&M University

Research Advisor: Dr. Clinton D. Allred
Department of Nutrition and Food Sciences

Coffee contains many bioactive compounds, one of which is trigonelline (TRG). Few studies have been performed to ascertain its effects on mammalian cells. However, TRG has been shown to have antimicrobial properties against enterobacteria, and has been found to increase formation of neurites in neuroblastoma cells. We were interested in determining whether trigonelline demonstrates estrogenic properties by activating the estrogen receptor. Estrogen dependent breast cancer (MCF-7) cells were used for cell proliferation assays to examine whether the compound is indeed estrogenic. In a dose response study, TRG stimulated MCF-7 cell proliferation. Even in doses as low as 100pM, cell growth was significantly increased. ICI, an estrogen receptor antagonist, inhibited TRG stimulated cellular proliferation in MCF-7 cells. Furthermore, co-treatment with estradiol and TRG stimulated MCF-7 cell growth to a greater extent than estradiol alone. Estrogen response element reporter assays were studied. These data establish that TRG promotes MCF-7 cell growth via the estrogen receptor and is estrogenic by nature. Therefore, trigonelline is a novel phytoestrogen.

DEDICATION

To my parents and my sister who have been a constant source of support, love, and encouragement in my life.

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Clinton Allred, for his invaluable support and assistance with this project. His guidance and mentorship have greatly impacted my experience as an undergraduate and encouraged me to further pursue research. I would also like to greatly thank Mrs. Kimberly Allred, who readily welcomed me into the laboratory and patiently guided me throughout the way.

NOMENCLATURE

TRG	Trigonelline
ER	Estrogen Receptor
ERE	Estrogen Response Element
DMEM + I	Dubleco's Modified Eagle's Media + Insulin
B-Gal	β -Galactosidase
NC	Negative Control

TABLE OF CONTENTS

	Page
ABSTRACT	iii
DEDICATION	iv
ACKNOWLEDGMENTS.....	v
NOMENCLATURE.....	vi
TABLE OF CONTENTS	vii
LIST OF FIGURES.....	viii
 CHAPTER	
I INTRODUCTION.....	1
II METHODS.....	5
Cells/reagents	5
Methods – cell growth and count	6
Methods – ERE luciferase and β -galactosidase assays	8
Statistics	9
III RESULTS.....	10
Trigonelline induces estrogen – dependent breast cancer cell growth.....	10
Co-treatment with estrogen and trigonelline results in an additive effect.....	11
Blocking ER fully inhibits TRG activity.....	12
Trigonelline activates ER in estrogen – dependent breast cancer cells.....	13
IV SUMMARY AND CONCLUSIONS.....	14
REFERENCES	16
CONTACT INFORMATION	17

LIST OF FIGURES

FIGURE	Page
1 MCF-7 Trigonelline Dose Response Cell Proliferation Assay.	10
2 MCF-7 Trigonelline/ Estrogen Combination Cell Proliferation Assay.	11
3 MCF-7 Trigonelline Cell Proliferation Assay Including ICI	12
4 ERE Luciferase / β -Galactosidase Assay	13

CHAPTER I

INTRODUCTION

Coffee is a widely consumed beverage in the United States and around the world. There are a number of biologically active compounds in coffee, such as caffeine, arabinogalactans, melanoidins, ash, organic acids, chlorogenic acid, and caffeic acid (1). Another of these chemical compounds present in coffee is trigonelline, which has been proven to have antimicrobial properties against enterobacteria. Trigonelline has been suggested to have multiple biological functions. Medicinally, trigonelline has been used as an antiseptic, antimigrane, and antitumor treatment (2). It has also been suggested that it could be used in foods as a natural preservative to control bacterial growth (3). Trigonelline is a biomarker; it contains intrinsic measurable biological activity. Trigonelline may aid in the regeneration of axons and dendrites to correct memory impairment (4). Trigonelline is a unique compound with many diverse properties. Our laboratory became interested in trigonelline because India, which has a long history of herbal supplements, employs trigonelline as a supplement for postmenopausal women. The objective of this research is to determine whether trigonelline is estrogenic, that is, it acts like an estrogen in the body. Specifically, this study will evaluate the effects of trigonelline on the growth of estrogen-dependent breast cancer cells.

This thesis follows the style and format of Cancer Research.

In 2006, there were an estimated 212,920 new cases of breast cancer, and 40,970 estimated deaths in the United States. Breast cancer is the number one cancer in women and comprises 31% of new cancer cases (5). It may develop due to genetic, dietary, or environmental factors, or a combination of the three. Breast cancer is most commonly diagnosed in postmenopausal women. There are two types of breast cancer: estrogen-dependent and estrogen-independent. With estrogen-dependent breast cancer, endogenous estrogen stimulates the growth of tumors. This is of particular concern in pre-menopausal women because estrogen levels are elevated. In postmenopausal women, where estradiol levels are low, there is worry about exposure to exogenous estrogen. Hormone replacement therapy is of key concern since the supplemented estrogen can also stimulate the growth of estrogen-dependent breast cancer in postmenopausal women. In addition, certain dietary components, such as soy (which contains phytoestrogens) and possibly coffee (which contains trigonelline), may stimulate the progression of estrogen-dependent breast cancer. Environmental estrogens and estrogenic compounds induce estrogen-dependent breast cancer cell growth. The identification and omission of estrogenic compounds in the diet of estrogen-dependent breast cancer patients may improve their medical treatment.

In general, a better understanding of breast cancer development and treatment is required to impact this disease. Many studies focus on tumor development. Diet clearly plays a role in influencing breast cancer formation. A diet high in alcohol, fat, and low in fiber, fruits, and vegetables is associated with high incidence of breast cancer (6). Fat and

alcohol intake are thought to be positively associated with risk of breast cancer because they increase endogenous estrogen (6). Also of importance is how dietary components influence already existing breast cancer tumors in women. It is estimated that it takes roughly 30 years for a tumor to develop and be detectable in many breast cancer patients. As such, many breast cancer patients will consume dietary compounds that may influence tumor growth even prior to diagnosis. So, understanding compounds that affect tumor progression is critical. Dietary compounds such as antioxidants and fiber are thought to inhibit breast cancer tumor growth (7). Research in the Allred laboratory is interested in determining compounds that may enhance breast cancer tumor growth, specifically, compounds that mimic estrogen and stimulate estrogen-dependent breast cancer tumor growth.

This research explores the compound trigonelline and the effects it has on breast cancer cell progression. One approach to explore breast cancer is *in vitro* assays. Breast cancer (MCF-7) cells were used to develop several key drug therapies, including tamoxifen and raloxifene (8). This model previously used for other dietary estrogens (isoflavones) will be used in our research to study trigonelline. To experimentally determine whether dietary estrogens possess estrogenic properties, the growth of estrogen-dependent breast cancer cells is observed under controlled conditions. These cells will grow more rapidly in the presence of estrogen and possibly trigonelline. For these studies, cell growth assays were conducted to monitor and measure growth patterns. Cell assays were also performed with estrogen receptor antagonists to block estrogen receptor activity and

evaluate whether the effects of the dietary estrogen compound were inhibited. When the estrogenic compound is not received in the receptor, it may not exert its influence on the cell. Then, an Estrogen Response Element (ERE) luciferase assay was conducted to evaluate whether or not trigonelline functions through the estrogen receptor (ER) which results in DNA transcription. A β -galactosidase assay was conducted as a housekeeping method to ensure equivalence across treatments.

The understanding gained from this study will further define the properties of trigonelline, and its effects on the human body. The study of trigonelline *in vitro* will improve understanding of its effects on pathophysiology. In the future, *in vivo* models are needed to further explore the estrogenic properties of trigonelline. The implications of this research may eventually influence and improve dietary modifications and recommendations for estrogen-dependent breast cancer patients.

CHAPTER II

METHODS

Cells / reagents

The estrogen – dependent breast cancer MCF-7 cell line was purchased from The American Type Culture Collection (ATCC). The cells are maintained in 10 mL Dubleco's Modified Eagle's Media, Sigma (DMEM) with the addition of 100 mL DMEM media, 500 μ L insulin (0.01 mg/ml insulin, Roche), 10% Fetal Bovine Serum, Hyclone (FBS), and 5 mL Penicillin/Streptomycin, Gibco (Pen/Strep). MCF-7 cells were stored in T75 flasks at 37°C with 5% CO₂.

DMSO was used as a control and was purchased from Sigma. E₂ (estradiol) was used for one set of treatments to compare to the trigonelline levels. Trigonelline was a gift from Jairam Vannmala. ICI was used as an inhibitor to estradiol and trigonelline in growth assays.

Methods – cell growth and count

Each experiment used one flask of cells. Old media was discarded and new media containing DMEM and Charcoal Dextran Stripped Fetal Bovine Serum (CDFBS) was added to the flask. After 3 days, media was removed from the cells and 1X Phosphate Buffered Saline (PBS) was added to the cells in addition to Trypsin EDTA. Trypsin was used to lift the cells from the surface of the flask. The cells were incubated for 5 min, and then tapped gently to make certain that all cells were released from the flask. Cells were then transferred to a 15 mL tube and centrifuged for 5 min at 800 X g. The cells were centrifuged to form a cell pellet. Then the media was removed from the tube and 5 mL fresh DMEM – CDFBS was added. The pellet was gently resuspended by pipeting up and down. Cells were then counted by transferring 10 μ L of the cell suspension to a hemacytometer.

150,000 cells in DMEM – CDFBS media were added to each well of four 6-well plates. A total of 3,600,000 cells were used in the experiment. The 6-well plates were gently tapped on each side to evenly distribute the cells in the wells. 24 h later, media was removed and the following treatments were added to the cells in DMEM-CDFBS media: Control (DMSO), 10 pM Estradiol (E_2), 1 pM Trigonelline (TRG), 10 pM TRG, 100 pM TRG, 10 pM E_2 + 1 pM TRG, 10 pM E_2 + 10 pM TRG, 10 pM E_2 + 100 pM TRG. Each of the stock solutions were made in DMSO, which served as a control. Stocks were made in order for a 1:1000 dilution to be made for each treatment to ensure consistency of volume between treatments.

Media was changed three days later. Then the same treatments were added to the cells again in DMEM-CDFBS media. After another 3 days, cells were prepared for counting on the coulter counter. Media was removed from each well. Then 100 μ L of 10 X Trypsin EDTA and 500 μ L 1X PBS were added to each well and incubated at 37°C for 5 min. When all cells were released from the plate, 1 mL of DMEM-CDFBS media was added to each well. The cells were mixed to remove clumps by pipeting media up and down gently. This also served to wash the well. The cells and media were transferred to a prelabeled 1.5 mL centrifuge tube. Cells tend to settle at the bottom of the tube, so they were then mixed by pipeting up and down. Then 40 μ L of the cell suspension was transferred to an accuvette containing 20 mL of Isoton II dilutant. To mix cells, the accuvette was capped and gently inverted 5 times. The coulter counter was set to read a 4 μ m cell size and the dilution factor was set at 1:500. An accuvette with only Isoton II dilutant served as the blank, and was placed on the coulter counter and read three times. Then a cell sample was placed on the coulter counter and read three times. The process of calibrating with a blank and reading a sample of cells was repeated for each treatment. Cell counts were recorded on a prepared excel spreadsheet. We made calculations for total cells per treatment well and averages were determined for each treatment. Graphs were then completed for cell averages with standard error. Each experiment was performed in triplicate for a sample size of $n=9$ for each treatment.

In subsequent cell growth experiments, identical experimental protocols were used.

However, for this study, treatments consisted of: Control, 100 pM E₂, 1 pM Trig, 10 pM Trig, 100 pM Trig, 1 μM ICI, 100 pM E₂ + 1 μM ICI, 100 pM Trig + 1 μM ICI.

Methods – ERE luciferase and β-galactosidase assays

Media was changed to DMEM+I+CDFBS. Three days later 60,000 cells / well in three 24-well plates were plated in striped serum media as above. 24 h later ESCORT – DNA mixture was prepared and treatments 500μL/well were added: DMSO, 1 μM ICI, 100 pM E₂, 1 μM ICI + 100 pM E₂, 100 pM Trig, 1 μM ICI + 100 pM Trig. Incubate 18 hr with treatments. Cells were lysed using reporter lysis buffer (RLB); 6 mL diH₂O + 1.5 mL RLB was needed. Media was aspirated off cells and cells were washed 2x with 1X PBS. 100 μL RLB was added and plates were rocked gently to completely cover cells. After 15 min incubation at RT, all areas of plate were scraped to loosen cells. Lysates were transferred to prelabeled 1.5 mL tubes and placed on ice. Tubes were vortexed 10 sec and spun @ 13,000xg for 2 min @ 40C. Next supernatant was transferred to a fresh (labeled) 1.5 mL tube. The Luciferase Assay (E1500 Promega) was performed according to manufacturer instructions. The plate reader was programmed for 2 sec delay; 10 sec read. 10 μL cell lysate / well was added and 50 μL Luciferase Assay Reagent / well was added immediately prior to reading. B-Gal Assay (E2000 Promega) was performed according to manufacturer instructions. The plate reader was programmed to read absorbance at 420nm. 25μl cell lysate + 20μl 2X assay buffer/well

was added. Plates were incubated at 37°C for 2 hours and a 75µl of Stop buffer was added and plates were immediately read on the plate reader.

Statistics

A one-way ANOVA hypothesis testing was conducted using StatTools 1.1 (Palisade, Ithaca, NY). In every case, significant differences were identified between various treatments within the cell line. Column graphs were used to display mean fold changes in luciferase ratios of treatments.

CHAPTER III

RESULTS

Trigonelline induces estrogen – dependent breast cancer cell growth

MCF-7 cells were dosed with varying treatments: a negative control, estradiol (E_2) and three doses of trigonelline (TRG) and evaluated on their growth. E_2 treated and TRG treated cells showed similar amounts of proliferation. Results showed that the 1 nM E_2 treatment had significantly more growth than the negative control, and trigonelline is dose responsive as seen in Figure 1. Similar to 1 nM E_2 , the 100 pM TRG treatment significantly induced cellular proliferation when compared to NC.

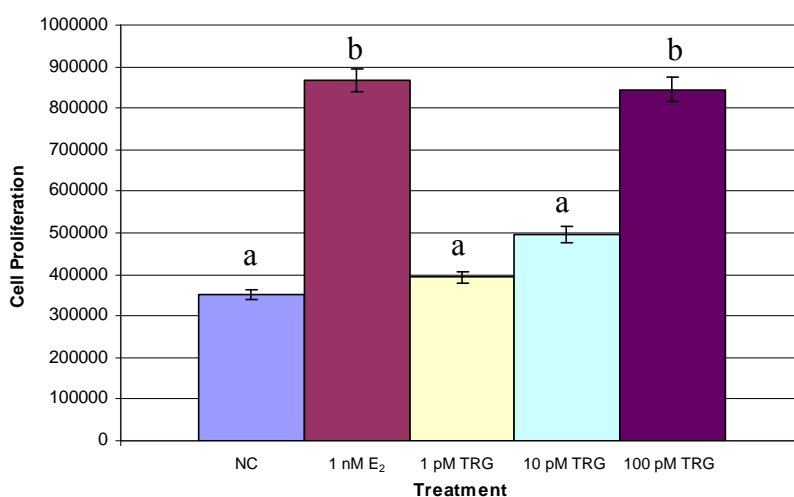


Figure 1. MCF-7 Trigonelline Dose Response Cell Proliferation Assay. Treatments were given for 3 days (2x). Three replicates were performed for n=9. The tallest column represents estrogen, while the second tallest column represents 100 pM TRG.

Co-treatment with estrogen and trigonelline results in an additive effect

When MCF-7 cells were co-treated with a low dose of E₂ and TRG, an additive effect was observed. 1 nM E₂ significantly stimulated growth compared to NC. A sub-optimal dose of estrogen was used for both the E₂ treatment and the co-treatment of E₂ and TRG.

When the cells were co-treated with 10pM E₂ + 100 pM TRG, significantly enhanced cellular proliferation effects were observed compared to the NC as seen in Figure 2.

Furthermore, co-treatment significantly enhanced growth when compared to E₂ treatment alone.

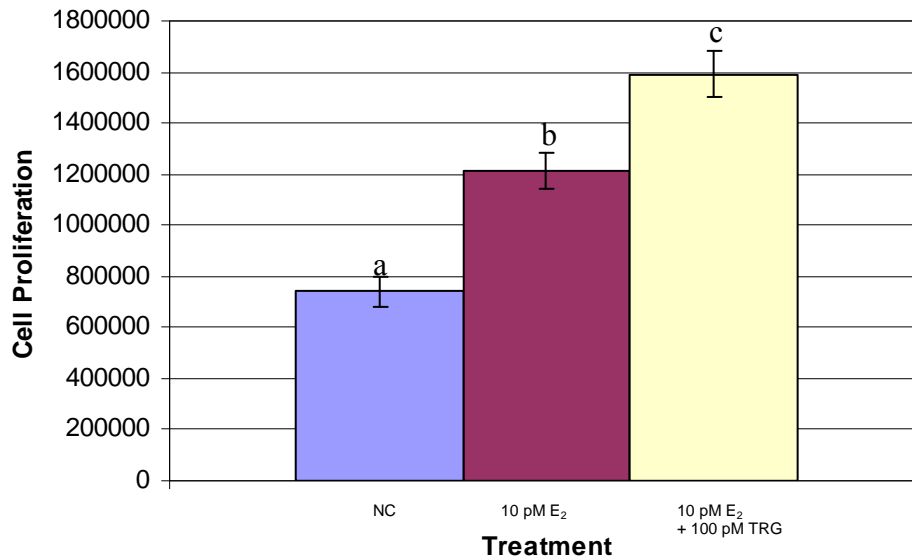


Figure 2. MCF-7 Trigonelline/ Estrogen Combination Cell Proliferation Assay. Treatments were given for 3 days (2x). Three replicates were performed for n=9. Cells treated with 10pM E₂ + 100 pM TRG showed greater proliferation than cells only treated with 10pM E₂.

Blocking ER fully inhibits TRG activity

ICI, which blocks the estrogen receptor (ER), fully inhibits estrogen activity as well as TRG activity in stimulating MCF-7 cell growth. The 100 pM E₂ treatment showed significantly more cellular proliferation than the negative control. As expected, ICI (an ER antagonist) completely inhibited estrogen-stimulated cell growth as seen in Figure 3. As before, 100 pM trigonelline stimulated cell growth and is significantly higher than the NC. ICI fully inhibited trigonelline's activity at 100 pM. The 100 pM E₂ treatment and 100 pM TRG treatments were not significantly different.

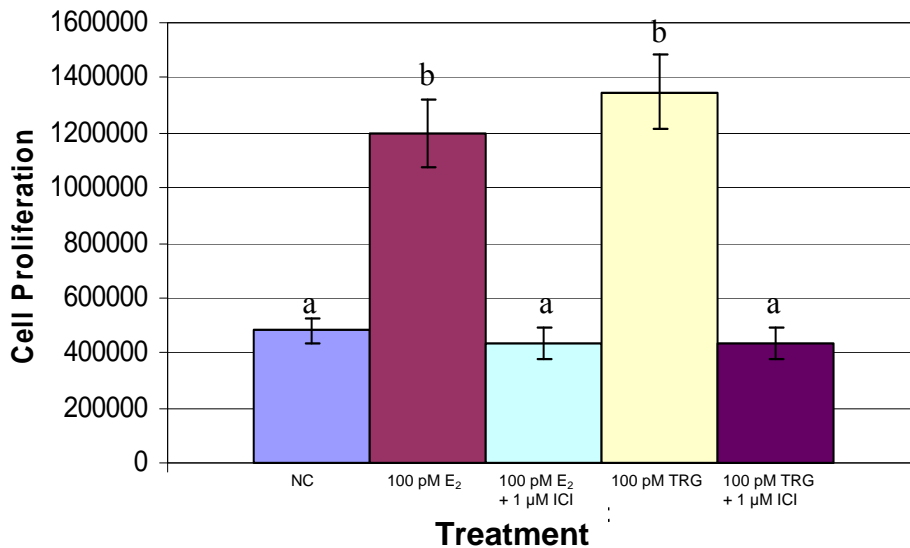


Figure 3. MCF-7 Trigonelline Cell Proliferation Assay Including ICI. Treatments were given for 3 days (2x). Three replicates were performed for n=9. When treated with ICI, Trig cells and E₂ cells behaved similarly, which demonstrates that Trig works through the estrogen receptor.

Trigonelline activates ER in estrogen – dependent breast cancer cells

In estrogen – dependent breast cancer cells, TRG increases cell growth via the estrogen receptor. We conducted an estrogen – response element luciferase reporter assay to measure DNA transcription and ERE activation. ICI alone had no effect. The transfected ERE lysates showed a three-fold increase for E₂ and a two-fold increase for trigonelline over the control and ICI inhibited treatments. In measuring the NC vs. 100 pM E₂, estrogen significantly increased activation of the ER in the ERE – reporter assay as seen in Figure 4. Additionally, 100 pM trigonelline significantly activated the ER compared to the NC.

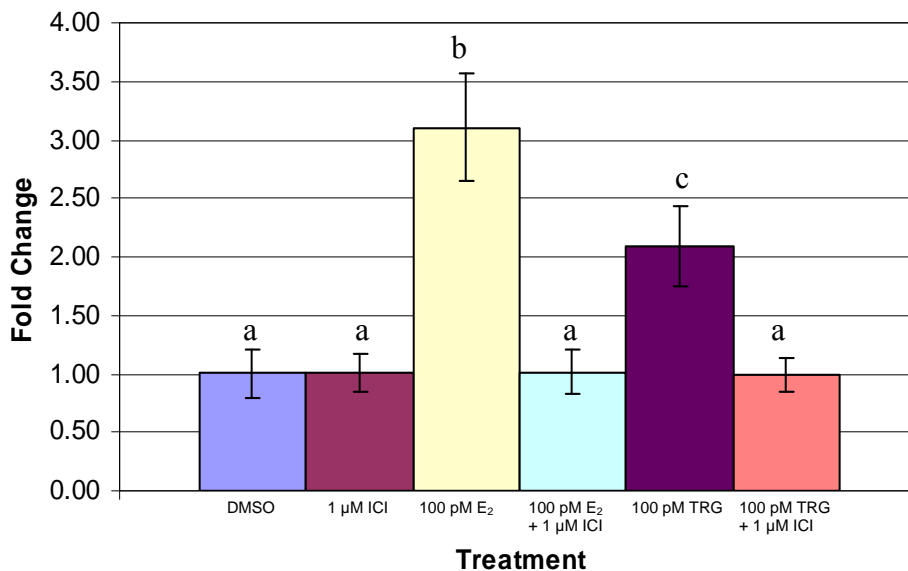


Figure 4. ERE Luciferase / β -Galactosidase Assay. Treatments were 18 h. The ERE reporter was used to show DNA transcription measured in luminescence. Three replicates were performed for the experiment; n=12.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Trigonelline is found in coffee. It has a few reported biological effects. We wanted to know if it has estrogenic properties and could be a new phytoestrogen. We used MCF-7 cellular proliferation *in vitro* as a model to initially screen for estrogenic activity. We found that trigonelline does increase MCF-7 cellular proliferation in a dose responsive manner. Treatment with trigonelline showed a significant increase in proliferation in the 100 pM dose. Trigonelline's response on the growth of MCF-7 cells did not demonstrate conclusively that it works through the estrogen receptor. Other compounds have been shown to be growth factors that stimulate MCF-7 proliferation through non-estrogenic mechanisms. Subsequent experiments investigated whether or not trigonelline functions via the estrogen receptor.

The study employing a sub-optimal dose of E₂ is potentially relevant to post-menopausal women with low estrogen levels circulating in the body. We wanted to test trigonelline (*in vitro*) in a low estrogen environment due to three important factors: the main group using phytoestrogens is comprised of post-menopausal women, they are most at risk for developing breast cancer, and phytoestrogens are most effective when estrogen is low or not present. Co-treatment of E₂ with TRG showed an additive effect in increased estrogen-dependent breast cancer cell proliferation.

Then we wanted to prove that trigonelline is estrogenic and works via the estrogen receptor on the cell surface. First, we used a cell proliferation assay with TRG + ICI. We found that ICI, a known ER inhibitor, fully inhibits cell growth effects of trigonelline. This is the first data to suggest that trigonelline utilizes the ER for its response. To confirm this, we employed ERE transcription reporter assays. This experiment studies whether or not trigonelline is capable of activating the ER which results in DNA transcription and activation of an ERE. Trigonelline significantly increased ERE activation as compared to NC. Our results clearly indicate that trigonelline is acting as a phytoestrogen.

In conclusion, it is not clear how much and how often exposure is necessary to obtain these results, nor is it evident how much TRG is present and bioavailable in coffee. Exposure to 100 pM doses of trigonelline is a potential problem for post-menopausal women with estrogen-dependent breast cancer. However, phytoestrogens are beneficial in other tissues. Additional research is needed to determine long-term health risks/benefits for trigonelline.

REFERENCES

1. Charles-Bernard M, Kraehenbuehl K, Rytz A, Roberts DD. Interactions between volatile and nonvolatile coffee components. 1. Screening of nonvolatile components. *J Agric Food Chem* 2005;53:4417-25.
2. Chopra S, Ahmad FJ, Khar RK, et al. Validated high-performance thin-layer chromatography method for determination of trigonelline in herbal extract and pharmaceutical dosage form. *Anal Chim Acta* 2006;577:46-51.
3. Almeida A. Antibacterial activity of coffee extracts and selected coffee chemical compounds against enterobacteria. *Journal of Agricultural and Food Chemistry* 2006;54:8738-43.
4. Tohda C, Kuboyama T, Komatsu K. Search for natural products related to regeneration of the neuronal network. *Neurosignals* 2005;14:34-45.
5. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2006. *CA Cancer J Clin* 2006;56:106-30.
6. Holmes MD, Willett WC. Does diet affect breast cancer risk? *Breast Cancer Res* 2004;6:170-8.
7. Michels KB, Mohllajee AP, Roset-Bahmanyar E, Beehler GP, Moysich KB. Diet and breast cancer: a review of the prospective observational studies. *Cancer* 2007;109:2712-49.
8. Okamoto Y, Liu X, Suzuki N, et al. Increased antitumor potential of the raloxifene prodrug, raloxifene diphosphate. *Int J Cancer* 2008;122:2142-7.

CONTACT INFORMATION

Name: Katarina Marie Yackley

Professional Address: c/o Dr. Clinton Allred
Department of Nutrition & Food Sciences
Texas A&M University
College Station, TX 77843

Email Address: kata04@tamu.edu

Education: BS Nutritional Sciences, Texas A&M University,
May 2008
Undergraduate Research Scholar